The role of CD151 and integrin 31 in the pathophysiology of kidney and skin
Sachs, L.N.

Citation for published version (APA):
Sachs, L. N. (2013). The role of CD151 and integrin 31 in the pathophysiology of kidney and skin

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
“The cell never acts; it reacts.”

Ernst Haeckel, 1866
Chapter 2

Kidney failure in mice lacking the tetraspanin CD151

Published in the Journal of Cell Biology
Kidney failure in mice lacking the tetraspanin CD151.

Norman Sachs¹, Maaike Kreft¹, Marius A. van den Bergh Weerman², Andy J. Beynon³, Theo A. Peters³, Jan J. Weening² and Arnoud Sonnenberg¹

¹ Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
² Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
³ Department of Otorhinolaryngology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

N. Sachs and M. Kreft contributed equally to this work.

Correspondence to Arnoud Sonnenberg: a.sonnenberg@nki.nl

Edited by Elaine Fuchs (Howard Hughes Medical Institute, Rockefeller University, New York, NY)

ABSTRACT
The tetraspanin CD151 is a cell surface molecule known for its strong lateral interaction with the laminin-binding integrin α3β1. Patients with a nonsense mutation in CD151 display end-stage kidney failure associated with regional skin blistering and sensorineural deafness and mice lacking the integrin α3 subunit die neonatally due to severe abnormalities in the lung and kidney epithelia. Here we report the generation of Cd151-null mice that recapitulate the renal pathology of human patients, i.e. with age they develop massive proteinuria due to focal glomerulosclerosis, disorganization of the glomerular basement membrane and tubular cystic dilation. However, neither skin integrity nor hearing ability are impaired in the Cd151-null mice. We furthermore generated podocyte-specific conditional knockout mice for the integrin α3 subunit, which show renal defects similar to those in the Cd151-knockout mice. Our results support the hypothesis that CD151 plays a key role in strengthening α3β1-mediated adhesion in podocytes.

INTRODUCTION
Tetraspanins form a family of small proteins that are expressed in virtually all cell types and tissues (Boucheix and Rubinstein, 2001; Hemler, 2005). They consist of short intracellular termini, four transmembrane domains, and one small and one large extracellular loop that contains two highly conserved cysteine motifs. Tetraspanins oligomerize into tetraspanin-enriched microdomains (TEMs) in which they associate with integrins, Ig superfamily members, growth factor receptors, and proteoglycans. TEMs modulate diverse cellular activities such as adhesion strengthening, migration, signal transduction and proliferation (Hemler, 2005). The importance of proper tetraspanin function is demonstrated by several human diseases: distinct mutations in A15 (TSPAN7) and RDS (TSPAN22) cause X-linked mental retardation and retinal dystrophy, respectively (Travis et al., 1989; Zemni et al., 2000). A nonsense mutation in CD151 (TSPAN24) leads to end-stage hereditary nephropathy associated with pretibial epidermolysis bullosa and sensorineural deafness (Karamatic Crew et al., 2004).

CD151 is expressed in epithelia, endothelia, muscle cells, renal glomeruli, proximal and distal tubules, Schwann cells, platelets, and dendritic cells (Sincock et al., 1997; Sterk et al., 2002).
Extensive biochemical studies have shown that CD151 is the primary tetraspanin associated with the laminin-binding integrins \( \alpha 3\beta 1, \alpha 6\beta 1, \alpha 6\beta 4, \) and \( \alpha 7\beta 1 \) (Boucheix and Rubinstein, 2001; Hemler, 2005; Sterk et al., 2000; Sterk et al., 2002). The interaction of CD151 with \( \alpha 3\beta 1 \) is particularly strong and occurs at high stoichiometry (Yauch et al., 1998).

Integrins are \( \alpha \beta \) heterodimeric cell surface proteins that dynamically link the extracellular matrix and/or adjacent cells to the intracellular cytoskeleton (Hynes, 2002; van der Flier and Sonnenberg, 2001). In epithelial cells the integrins \( \alpha 3\beta 1 \) and \( \alpha 6\beta 4 \) are mainly present in the basolateral compartment where they bind to the basement membrane component laminin-5. Although much more severe, the phenotypes associated with mutations in \( Itga3, \) \( Itga6, \) and \( Itgb4 \) share features with the phenotype of patients with truncated CD151, indicating that the CD151-\( \alpha 3\beta 1 \) and CD151-\( \alpha 6\beta 4 \) heterotrimers are functionally important. Mice that lack the \( \beta 4 \) subunit suffer from extensive detachment of the epidermis and patients without functional \( \alpha 6\beta 4 \) display junctional epidermolysis bullosa (Borradori and Sonnenberg, 1999; Uitto and Pulkkinen, 2001). Mice without \( \alpha 3 \) exhibit mild skin blistering associated with ruptured basement membranes and die shortly after birth due to severe abnormalities in the epithelia of lung and kidney (DiPersio et al., 1997; Kreidberg et al., 1996).

In the renal glomerulus, podocytes are anchored to the glomerular basement membrane (GBM) via \( \alpha 3\beta 1 \) and dystroglycans (Mundel and Shankland, 2002). The interdigitating foot processes (FPs) of podocytes are connected by glomerular epithelial slit diaphragms (GESDs) consisting of nephrin, podocin, P-cadherin and other proteins, linked directly or indirectly to the cytoskeleton. Both disturbed podocyte-GBM anchoring and podocyte-podocyte interaction at the level of GESDs, lead to the loss of FPs, a dysfunctional filtration barrier and ultimately to glomerulosclerosis and renal failure (Pavenstadt et al., 2003).

Here we report the generation of knockout mice for \( Cd151 \) which show severe renal failure due to progressive abnormalities of the GBM, loss of podocyte FPs, glomerulosclerosis and cystic tubular dilation. We furthermore show that mice with a targeted deletion of the \( \alpha 3 \) subunit in podocytes have a similar though more severe phenotype

**RESULTS AND DISCUSSION**

\( Cd151 \)-null mice were generated (Supplemental Figure 1), born at the expected Mendelian ratio (0.28 \( (+/+): 0.49 \) \( (+/-): 0.23 \) \( (-/-): \) n=86) and appeared to be healthy and normal at first observation. However, urine analysis of \( Cd151^{+/+} \) mice by SDS-PAGE revealed that all mice developed proteinuria before 3 weeks of age indicative of kidney dysfunction (Figure 1A).

Both the onset and the degree of proteinuria were variable. Nevertheless, all knockout mice had to be sacrificed before the age of 9 months because of significant loss of body weight. A quantitative immunoassay of urinary albumin showed high levels of this protein in \( Cd151^{+/+} \) mice that increased with age and reached a plateau at 6 weeks (3 months follow up, data not shown). In contrast, only traces of albumin were present in the urine of wild-type and \( Cd151^{+/+} \) mice (Figure 1B,C). As expected the severity of the renal pathology among \( Cd151^{+/+} \) littermates also varied considerably (Figure 2A,B) and there were animals with mildly or severely affected
kidneys in the same litter. Histological examination of the mildly affected kidneys showed focal glomerulosclerosis, and interstitial fibrosis and inflammation (Figure 2D,G). The GBM of some capillary loops was abnormally thick and formed extensive spikes (Figure 2J).

Electronmicroscopy revealed that the GBM was laminated and that FPs in contact with the abnormal GBM were effaced (Figure 3A-C). On the vascular side the endothelium was swollen and fenestrations were occasionally lost (Figure 3B,C). Severely affected kidneys were contracted and their capsules granulated due to cortical degeneration (Figure 2B). Light microscopy showed extensive glomerulosclerosis in several stages, tuft adhesions to Bowman’s capsule with extracapillary cell proliferation and fibrosis, and marked expansion of the mesangial matrix. Proximal tubuli were either dilated and contained PAS-positive protein casts or displayed degeneration of different severity (Figure 2E,H). We furthermore observed periglomerular fibrosis and focal interstitial inflammation in close proximity to interlobular blood vessels (data not shown). Silver staining showed the glomeruli to be segmentally or globally sclerosed with extensive deposits of basement membrane components (Figure 2K), an observation that could be confirmed by ultrastructural analysis (data not shown). To investigate at what age GBM defects occur we subjected kidneys of newborn and 1 week old

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cd151 wild-type</th>
<th>2.5P-Cre; Itga3fl/fl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1/9</td>
<td>1/6</td>
</tr>
<tr>
<td>1</td>
<td>2/9</td>
<td>4/6</td>
</tr>
<tr>
<td>2</td>
<td>1/9</td>
<td>1/6</td>
</tr>
<tr>
<td>3</td>
<td>5/9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1

Cd151<sup>−/−</sup> and 2.5P-Cre; Itga3<sup>fl/fl</sup> mice reveal massive albuminuria.

(A) Age at which proteinuric was first detected by SDS-PAGE in Cd151<sup>−/−</sup> and 2.5P-Cre; Itga3<sup>fl/fl</sup> mice.
(B) Twenty-four-hour urinary albumin concentration in 6 week old mice as determined by competitive ELISA. Each bar represents the arithmetic mean ± SD with P values as determined by homoscedastic two-tailed Student’s t-Test shown above and the number of analyzed animals shown underneath.
(C) Commassie brilliant blue stained gel showing the presence of albumin in the urine of Cd151<sup>−/−</sup> mice in a group of 3 litters (1 μl urine per lane; age: 6 weeks).
Figure 2 Kidneys of Cd151^−/− and 2.5P-Cre; Itga3^fl/fl mice reveal mild (left column) and severe (middle and right columns) renal pathology.

Although mildly affected kidneys of a 3 month old Cd151^−/− mouse show a relatively smooth and intact surface (A), histological examination reveals protein casts (D, arrow), inconspicuous interstitial fibrosis, mesangial hypercellularity (G, open arrowhead), and mild glomerular sclerosis (G, open arrow). The GBM shows partial thickening and spike-formation (J, inset). Severe lesions of another 3 month old Cd151^−/− mouse are characterized by a granular surface (B) due to extensive glomerular collapsing sclerosis, periglomerular fibrosis and focal interstitial inflammation and fibrosis (E, H, K). The glomeruli show segmental (H, 1) and global (H, 2) attachment of glomerular tufts to Bowman’s capsule, parietal epithelial crescents (H, 2), and hypercellularity, capillary collapse and sclerosis (H, 3). Proximal tubuli show cystic dilation (E, arrowheads) and degeneration as well as protein casts indicative of proteinuria (E, H, arrows). Silver staining indicates excessive GBM damage in some glomeruli (K). Severely affected kidneys of a 6 week 2.5P-Cre; Itga3^fl/fl mouse reveal prominent protein casts and some extremely dilated proximal tubuli (F, I, arrows). The glomeruli are partially sclerosed and show substantial damage of the GBM (L). Scale bar: 50 μm
Figure 3 Electronmicrographs reveal GBM abnormalities and FP effacement in kidneys of mildly affected Cd151<sup>−/−</sup> and 2.5P-Cre; Itga3<sup>fl/fl</sup>, but not in 2.5P-Cre; Itga3<sup>+/fl</sup> mice. (A) Glomeruli of Cd151<sup>−/−</sup> mice (3 month old) show abnormal capillary loops characterized by GBM thickening and loss of the regular pattern of podocyte FPs. (B) Peripheral glomerular capillary with a luminal thrombocyte and loss of fenestrations (arrowhead). The GBM is irregularly thickened and shows protrusions towards the capsular space (*). Podocyte FPs are partially lost (arrows). (C) Irregular GBM showing, lamination, thickening, and protrusions (*). The endothelium is swollen and fenestrations are partially lost (arrowheads), so are podocyte FPs (arrows). Glomeruli of 6 week old 2.5P-Cre; Itga3<sup>+/fl</sup> mice do not show abnormalities (D), whereas all glomeruli of 2.5P-Cre; Itga3<sup>fl/fl</sup> mice exhibit severe structural changes (E, F). (E) Abnormally thickened GBM with protrusions throughout the glomerulus. (F) Podocyte FPs are completely lost (arrows) and GBM protrusions (*) are present along the entire length of the capillary loops. Solid lines point to normal structures (GBM, FPs), while dotted lines indicate abnormal thickening of the GBM and effacement of FPs. Bc: Bowman’s capsule, Cs: capsular space, E: erythrocyte, Ec: endothelial cell, Fp: foot process, Lb: lamina basalis (GBM), Pdc: podocyte, T: thrombocyte, V: vascular lumen; scale bar: 10 μm (A, D, E), 2 μm (B, C, F)
Cd151-null mice to electron microscopy. Although many capillary loops of newborn Cd151−/− mice already have a laminated GBM, it seems that spike formation does not occur until the mice are 1 week old (data not shown). Together, these observations suggest that the mild abnormalities found in some of the mice represent early stages of the severe phenotype in the other mice. Tubular changes may be secondary to massive glomerular protein leakage but may also reflect dysfunction of the tubules themselves.

To investigate whether the progressive GBM abnormalities are correlated with glomerular injury, we stained for tenascin-C and fibulin-2. Both proteins have been shown to be upregulated in response to glomerular and vascular lesions (Assad et al., 1993; Wada et al., 2001). We observed an increased glomerular expression of tenascin-C in both mildly and severely affected kidneys, but fibulin-2 was upregulated only in the latter (Figure 4A-C). Whereas fibulin-2 is important for the migration of smooth muscle cells, tenascin-C regulates migration of a variety of cell types including fibroblasts (Hsia and Schwarzbauer, 2005; Strom et al., 2006). The finding that tenascin-C is already present in the early stages of the disease suggests that fibrosis precedes the pathology of the vasculature. Consistent with the ultrastructural finding that the GBM of Cd151−/− mice is thickened, staining for nidogen and laminin-10 revealed an abnormally strong presence of these GBM components in peripheral capillary loops and extracapillary spaces (Figure 4K-M, P-R). In order to exclude an arrest of the normal developmental switch from α1.α1.α2 (IV) collagen to the α3.α4.α5 (IV) and α5.α5.α6 (IV) collagen networks as seen in the X-linked form of Alport’s syndrome (Kalluri et al., 1997), we stained for all 6 chains of collagen IV. The results showed the mature collagen IV pattern to be present in all glomeruli (data not shown). Only the α2 (IV) chain was strongly upregulated in podocytes of the severely affected kidneys (Figure 4W). We furthermore checked the integrity of the filtration barrier by staining for the GESD components podocin and nephrin (Figure 4P-R, U-W). Both proteins appeared to be downregulated in the mildly affected kidneys and are almost absent in the severely affected kidneys, demonstrating a complete loss of GESD architecture (Figure 4R,W). Together these results support the hypothesis that in Cd151-null mice disorganization of the GBM precedes the effacement of FPs and the loss of GESDs. Patients with a nonsense mutation in CD151 (Karamatic Crew et al., 2004) also have epidermolysis bullosa and deafness, but neither of these was observed in the Cd151−/− mice by histological and immunofluorescent analysis and measurements of auditory brainstem responses (ABR), respectively (Supplemental Figure 2).

The highly stoichiometric binding of CD151 to the α3β1 integrin and the fact that mice lacking the α3 subunit show severe renal defects, led us to hypothesize that the absence of CD151-α3β1 complexes is responsible for the renal pathology seen in our Cd151-null mice. In Itga3 knockout mice glomerular capillary branching is impaired, leading to a decreased number of capillary loops. Furthermore, podocytes fail to form normal FPs and lose lateral cell junctions (Kreidberg et al., 1996). To study the deletion of Itga3 after glomerular capillary branching and to investigate possible similarities with the phenotype in our Cd151-null mice, we generated conditional Itga3-knockout mice and crossed them with 2.5P-Cre mice that express the Cre recombinase under the control of the human podocin promoter (Moeller et al., 2003).
As shown by immunofluorescence α3 is indeed almost absent in the glomeruli of these 2.5P-Cre; Itga3^fl/fl mice (Figure 4I,J), indicating that this integrin subunit is mainly expressed by differentiated podocytes. 2.5P-Cre; Itga3^fl/fl mice show massive proteinuria starting within the first week of age (Figure 1A,B), develop abdominal edema when 5 to 6 weeks old and have to be sacrificed subsequently. Structurally the milky discolored kidneys contain partially sclerosed glomeruli with a disorganized GBM and prominent protein casts in dilated proximal tubuli (Figure 2). Electron microscopy revealed a complete effacement of podocyte FPs in newborns (data not shown) and in addition widespread lamination and protrusions of the GBM in 6 week old mice (Figure 3E,F). Immunofluorescence showed changes similar to those in Cd151^–/– mice, i.e. upregulation of tenascin-C and fibulin, strong staining of the GBM components nidogen and laminin-10, and downregulation of the GESD proteins podocin and nephrin (Figure 4). Notably, sclerosis of the glomeruli, upregulation of the collagen α2 (IV) chain and downregulation of podocin and nephrin is much less prominent in the 2.5P-Cre; Itga3^fl/fl mice than in the Cd151 knockout. 2.5P-Cre; Itga3^+/fl mice do not develop structural or functional renal abnormalities (Figure 1A, 3D, 4), neither do 6 week old compound heterozygotes (2.5P-Cre; Itga3^+/fl; Cd151^+/–), which may suggest that a simultaneous reduction of α3 and CD151 does not result in renal defects (Figure 1C). However, we cannot prove that the expression of these proteins is actually reduced in the compound heterozygotes and neither can we exclude that renal pathology develops upon aging.

Since Cd151-null mice can form normal GBMs with a regular FP pattern (Figure 3A) and expression of both α3 and α6 in the glomeruli and tubules appeared to be normal (Figure 4F-H), we suggest that the function of α3 in development is not affected by the absence of CD151. Instead we propose that reduced integrin α3β1-mediated adhesion is the main cause of the phenotype observed in our Cd151^–/– mice. The filtration of plasma exerts considerable mechanical stress on the filtration barrier. Podocyte FPs thus have to withstand substantial mechanical forces. Indeed, CD151 appears to be involved in adhesion strenghtening, since adhesions mediated by CD151-α6β1 complexes tolerate stronger mechanical forces than those mediated by α6β1 alone (Lammerding et al., 2003). A similar effect has been suggested for complexes of CD151 and α3β1 (Nishiuchi et al., 2005). FPs without CD151 might thus not be able to withstand prolonged transcapillary pressure, a phenomenon that is also responsible for the renal manifestations in the Alport’s syndrome after several years of life in patients with an abnormal GBM due to collagen type IV mutations (Kalluri et al., 1997). Epithelial cells may become partially detached leading to a compensatory production of new basement membrane components. As a result, the GBM thickens and spikes are formed, as has also been described in membranous nephropathy (Minto et al., 1998). Correct reassembly of basement membranes upon injury is impaired in the skin of Cd151-null mice (Cowin et al., 2006). If the absence of CD151 is similarly important for GBM repair, this vicious circle would indeed result in glomerular malfunction. As shown by ultrastructural and immunofluorescent analysis, glomerulosclerosis precedes the loss of GESD components leading to the described renal pathology.
Figure 4 Immunofluorescence analysis of kidneys from 3 month old wild-type (left column), mildly and severely affected Cd151<sup>−/−</sup> (second and third column), as well as from 6 week old 2.5P-Cre; Itga3<sup>+/fl</sup> and 2.5P-Cre; Itga3<sup>fl/fl</sup> mice (columns four and five). The respective proteins are shown in green and red, yielding yellow upon colocalization. Nuclei are counterstained with TOPRO (blue). (A-C) Upregulation of tenascin-C in the mildly, and of fibulin-2 in the severely affected Cd151<sup>−/−</sup> glomeruli. (D, E) 2.5P-Cre; Itga3<sup>+/fl</sup> glomeruli show little presence of tenascin-C, whereas both tenascin-C and fibulin-2 are present in glomeruli of 2.5P-Cre; Itga3<sup>fl/fl</sup> mice (F-I) Integrin α3 and α6 are localized in glomeruli and tubules, respectively in both wild-type and mildly affected Cd151<sup>−/−</sup> kidneys as well as in the kidneys of 2.5P-Cre; Itga3<sup>+/fl</sup> mice. (J) Hardly any α3 is present in the glomeruli of 2.5P-Cre; Itga3<sup>fl/fl</sup> mice, whereas α6 is normally distributed in the tubules. (K, N) Staining of nidogen shows regular GBMs in both wild-type and 2.5P-Cre; Itga3<sup>+/fl</sup> mice. (L, M) Unusually strong nidogen staining of the GBM of peripheral capillary loops in both mildly and severely affected Cd151<sup>−/−</sup> kidneys (arrowheads). Note the extracapillary accumulation of nidogen (arrow) and massive capillary collapse (b1 staining) in the severely affected kidneys. (O) Nidogen also accumulates in the capsular space of 2.5P-Cre; Itga3<sup>+/fl</sup> mice (arrow). Glomeruli are not collapsed but show an abnormally thick GBM (arrowhead) with protrusions (open arrow). (P-T) Podocin is present along the glomerular tuft in wild-type, mildly affected Cd151<sup>+/−</sup> and 2.5P-Cre; Itga3<sup>+/fl</sup> mice, but absent in severely affected Cd151<sup>−/−</sup> and 2.5P-Cre; Itga3<sup>fl/fl</sup> mice. Staining of laminin-10 shows sclerosed glomeruli in Cd151<sup>−/−</sup> mice (R) and GBM thickening (arrowhead) with protrusions (open arrow) in 2.5P-Cre; Itga3<sup>fl/fl</sup> mice (T). (U-W) Nephrin is lost in some glomeruli of mildly affected Cd151<sup>−/−</sup> mice but in all glomeruli of the severely affected Cd151<sup>−/−</sup> mice. There is massive upregulation of collagen α2 (IV) in the severe Cd151<sup>−/−</sup> phenotype (W) which is furthermore characterized by hypercellularity in both the interstitium and glomeruli (C, H, M, R, W; blue channel). (X, Y) Nephrin is also reduced in 2.5P-Cre; Itga3<sup>fl/fl</sup> mice as compared to the conditional heterozygote, whereas the collagen α2 (IV) is hardly upregulated. Insets K-O: green channel (nidogen); Inset T: red channel (laminin-10); scale bar: 50 μm.
The assumption that CD151 is important for maintaining glomerular architecture upon mechanical stress is in accordance with the observation that glomeruli develop normally and that abnormalities only occur after several weeks or months. Furthermore, it explains differences in the rate of progression among littermates since the degree of intraglomerular hydrostatic pressure depends on several genetic and epigenetic factors. Differences in genetic background might also explain why Cd151-null mice that have been described previously, did not show renal failure as observed in our mice (Wright et al., 2004). In conclusion, we show that the renal manifestations in our mice lacking CD151 are similar to those in patients with a mutated CD151. Our data furthermore support in vitro studies pointing to a role of CD151 in adhesion strengthening, thus suggesting that CD151-α3β1 complexes have an essential function in vivo.

MATERIALS AND METHODS
Generation of Cd151-knockout mice
A BAC clone comprising exons 1 to 9 of Cd151 was isolated from a 129S6/SvEvTac library (RPCI-21, Invitrogen). An 8.8 kb fragment of genomic Cd151 was cloned in three steps into pFlexible, a generic targeting vector containing the selectable marker puroDtk and loxP and frt recombination sites (van der Weyden et al., 2005) using sequence specific primers containing restriction site tags. Fragment Cd151 I was amplified with Pwo polymerase using primers P4 plus P5. Primers for the amplification of Cd151 II and Cd151 III were P6 plus P7 and P8 plus P9, respectively (Table 1). After linearization with Pmel and NotI, 80 μg of the target construct were electroporated into 129/Ola-derived ES cells. Colonies resistant to 3.3 μM puromycin were screened for the desired homologous recombination by Southern blotting using a 5’ specific probe designed with primers P10 and P11. The puroDtk cassette flanked by frt sites was removed by transient transfection of pFLPe (Rodriguez et al., 2000). Colonies resistant to 5 μM ganciclovir were selected and exons 2-4 of Cd151 were subsequently deleted by transient transfection of a Cre-expression plasmid pOG231 (O’Gorman and Wahl, 1997) (Supplemental Figure 1A,B). One recombinant ES cell clone harboring the Cd151 null allele was injected into mouse C57Bl/6 blastocysts, which were transferred to mothers of the same strain. The chimeric male offspring was mated with FVB/N females. Agouti-coat-colored offspring was screened for the absence of exons 2-4 with by PCR analysis of tail DNA with primers P1-P3 (Table 1, Supplemental Figure 1C). Heterozygous mice were intercrossed and littermates were analyzed. The absence of Cd151 was verified by immunoblotting lysates of Cd151+/− mouse embryonic fibroblasts (MEFs) (Supplemental Figure 1D), which were prepared from embryos at day 13.5 post coitum.

Generation of podocyte specific Itga3-knockout mice
Itga3△/△ mice, which were generated by flanking exon one of the integrin α3 gene with two loxP sites, were crossed with 2.5P-Cre transgenic mice (Moeller et al., 2003) and Cd151-null mice to produce animals with a podocyte-specific deletion of the α3 subunit, alone or in combination with the deletion of Cd151, respectively. All animal experiments were carried out with approval from the relevant institutional animal ethics committees.
Urine analysis
Urine from mice younger than 3 weeks were collected by applying gentle dorsal pressure to the caudal area of the animal. Older mice were placed in metabolic cages for 24h. Samples were either analyzed by SDS-PAGE followed by Coomassie Brilliant blue staining or by competitive ELISA using the Albuwell M kit from Exocell (Philadelphia, PA).

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Sequence (5' to 3')</th>
<th>Restriction sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GTCTGACCACCCCATTCATTGTC</td>
<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>GCATGCTGCCTCAGTGAAGC</td>
<td>-</td>
</tr>
<tr>
<td>P3</td>
<td>GGTGCCACGAAATGTCTCTCCA</td>
<td>-</td>
</tr>
<tr>
<td>P4</td>
<td>GGCAGCCGCCTCAGGATTCGGAGGATGTA</td>
<td>Ascl</td>
</tr>
<tr>
<td>P5</td>
<td>GGCAGCGAGGATCC AAAGACATCAACAGACTGGATGTA</td>
<td>BamHI / Ascl</td>
</tr>
<tr>
<td>P6</td>
<td>CCTTAATAGCTGGCTAGGATGAATGTC</td>
<td>PacI</td>
</tr>
<tr>
<td>P7</td>
<td>CCTTAATTAAGGGTGTAGAGGGATGTG</td>
<td>PacI</td>
</tr>
<tr>
<td>P8</td>
<td>CCTGAGGGATATAGTA GTGAGGATGTC</td>
<td>EcoRV / SbfI</td>
</tr>
<tr>
<td>P9</td>
<td>CCTGAGGTCGTGCTATGGTCAGAATGGAGGATGTC</td>
<td>SbfI</td>
</tr>
<tr>
<td>P10</td>
<td>GTATACTGTGTACCTCTTGTC</td>
<td>-</td>
</tr>
<tr>
<td>P11</td>
<td>AGAGGTCCACAGACTGAGT</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1 Primers used in this study
Shown are Cd151 sequence specific primers with restriction site tags (italic) used for cloning of Cd151-fragments into pFlexible (P4-P9), for generating the 5'-Southern blot probe (P10, P11), and for PCR analysis of mouse tail DNA (P1-P3).

Immunoblotting
Mouse embryonic fibroblasts were lysed in Nonidet P-40 lysis buffer [1% (vol/vol) Nonidet P-40, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 4 mM EDTA] containing a cocktail of protease inhibitors (Sigma-Aldrich Chemie B.V., Zwijndrecht, NL). Lysates were clarified by centrifugation at 20,000 x g for 20 minutes at 4°C. Aliquots of cell lysates containing equal amounts of proteins were subjected to SDS-PAGE on a 12% polyacrylamide gel under non-reducing conditions, followed by transfer to Immobilon-PVDF membranes (Millipore Corp., Bedford, MA). The membranes were blocked and blots subsequently developed with the indicated antibodies using the ECL detection kit (ECL, Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

Histological analysis
Sections of kidneys were prepared, fixed for one day in EAF (ethanol, acetic acid, formaldehyde) and stained with PAS-D, HE or Jones’ methenamine silver. Images were taken with Zeiss PL APO objectives (10x/0.25, 40x/0.95, 63x/1.4 oil) on an Axiovert S100 / AxioCam HR color system using AxioVision 4 software (Carl Zeiss MicroImaging GmbH, Jena, Germany).
Ultrastructural analysis
After fixation in Karnovsky buffer for 48 hours, the material was post-fixed with 1% osmiumtetroxide, the tissue samples were block-stained with 1% uranyl acetate, dehydrated in dimethoxypropane and embedded in epoxyresin LX-112. LM sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate and lead citrate and examined using a Philips CM10 transmission electron microscope (FEI). Images were acquired using a digital transmission EM camera (Morada 10-12, Soft Imaging System, RvC, Soest, NL) using the software Research Assistant (RvC, Soest, NL).

Antibodies
Rat mAbs used in this study were 4G6 against laminin-10 (kindly provided by Dr. L. Sorokin, University of Münster, Münster, Germany), GoH3 against α6, MB1.2 against β1 (a kind gift from Dr. B.M. Chan, University of Western Ontario, London, Canada), LAT-2 against tenascin-C (van der Flier et al., 1997) and 346-11A against β4 (Abcam, Cambridge, UK). Dr. Y. Sado (Shigei Medical Research Institute, Yamada, Japan) kindly provided the rat mAbs H11, H22, H31, RH42, M54, and B66 against the mouse collagen IV chains α1, α2, α3, α4, α5, and α6, respectively. Rabbit polyclonal antibodies (pAbs) directed against mouse nidogen and mouse fibulin-2 were generous gifts from Dr. T. Sasaki (Max Planck Institute for Biochemistry, München, Germany); pAbs against nephrin and podocin were from Dr. H. Holthöfer (University of Helsinki, Helsinki, Finland) and Dr. C. Antignac (Cochin Biomedical Research Institute, Paris, France). The pAbs against keratin 1 and 14 were purchased from BabCO (Berkely, CA). Immunization of New Zealand rabbits with the cytoplasmic tail of human α3A fused to GST and the peptide CKENLKDVMKRYHQSGHEGVSSAVDKLQQEFH coupled to KLH (Pierce Biotechnology Inc., Rockford, IL) yielded pAbs 141742 against α3A and 140190 against murine CD151, respectively. Texas Red and fluorescein isothiocyanate (FITC) conjugated secondary antibodies were from Molecular Probes (Eugene, OR).

Immunofluorescence microscopy
Tissues from adult mice were collected and embedded in cryoprotectant (Tissue-Tek® O.C.T., Sakura Finetek Europe, Zoeterwoude, NL). Cryosections were prepared, fixed in ice-cold acetone, blocked with 2% BSA in PBS and incubated for 45 min with primary antibodies undiluted (LAT-2, GoH3, MB1.2, 4G6) or diluted 1:2 (H22), 1:50 (346-11A), 1:100 (anti-fibulin-2, anti-podocin, anti-nephrin, 141742), 1:250 (anti-nidogen), 1:300 (anti-keratin 1 and 14) followed by incubation with secondary antibodies diluted 1:200 for 45 min. Samples were analysed at 37°C using a 63x/1.4 HCX PL APO CS objective on a TCS SP2 AOBS confocal microscope (Leica, Mannheim, Germany). Images were acquired using LCS 2.61 and processed using CorelDRAW® Graphics Suite 12.

Auditory brainstem response (ABR) measurements
ABR measurements were performed in a sound-proof room with low reverberation. Needle electrodes were placed on M1 and M2 (left and right mastoids) and referred to the fronto-central midline (vertex, Cz) to record the auditory-evoked potentials. A ground electrode was placed halfway on the tail of the mice. Interelectrode impedances were measured before and
after each measurement (< 8 kOhm). Click stimuli of 100 μs and tone burst stimuli of 8, 16 and 32 kHz (1 ms rise/fall, 3 ms plateau time) were presented in a sound field by placing the loudspeakers 5 cm in front of each ear. The loudness levels at the position of the ear were measured and calibrated with a Brüel and Kjaer 2203 sound pressure level (SPL) meter. All thresholds were corrected afterwards for the soundfield setup. Before the measurements were performed, the mice were i.p. injected with ketamine (200 mg/kg) anesthetic. Stimuli were presented with a fixed stimulation rate of 32 Hz and a standard-auditory evoked potential recording system (Synergy, Oxford Instruments) was used to record the ABRs. The analysis time was set at 15 msec from the onset of the click with a 1.5 msec prestimulus time to assess baseline levels. The recorded EEG signals were high-pass filtered at 100 Hz and low-pass filtered at 3 kHz; an automatic artifact rejection and a 60 Hz notch filter were used to avoid EMG or external noise. Auditory brainstem responses were obtained from both contra- and ipsilateral stimulation sites. The EEG signals were averaged for different stimulation levels according to standard audiometrical top-down procedures, starting at 90 dB (SPL), uncorrected for the soundfield. Peaks were identified according to the Jewett and Williston nomenclature (Jewett and Williston, 1971). The auditory hearing threshold was defined as the lowest level (in dBSPL) at which at least one reproducible peak was visually recognized in the responses obtained from the ipsilateral measured ear. Between-group ABR thresholds differences of click- and high-frequency tone bursts were determined and analyzed for the control, the Cd151−/− and the Cd151+/+ mice using analysis of variance by the Bonferroni statistics (SPSS, vs 12.0.1).

ACKNOWLEDGEMENTS
We are grateful to Dr. L. B. Holzman for generously providing the 2.5P-Cre transgenic mice, to Drs. C. Antignac, A. Bradley, B. Chan, B. Lane, H. Holthofer, Y. Sado and T. Sasaki for providing reagents. We would like to thank Dr. K. Wilhelmsen and I. Kuikman for the generation of antibodies against Cd151 and the integrin α3 subunit. This work was supported by grants from the Dutch Cancer Society and the Netherlands Kidney Foundation.

REFERENCES


Supplemental Figure 1 Targeting strategy and molecular analysis of recombinant ES cells and Cd151-knockout mice.

(A) Cd151 gene structure, targeting construct, and different Cd151 mutant alleles. Numbered grey and black boxes represent noncoding and coding exons, respectively. Grey and black triangles mark frt and loxP sites. Cd151 fragments I, II, and III along with their bp positions and restriction sites used to generate the targeting construct are indicated. Shown are the locations of EcoRV cleavage sites (bold), along with a hybridizing probe, and primers (arrows) that were used for the analysis of the different mutant alleles by southern blotting and PCR, respectively. Dashed and dotted lines indicate the FLPe- and Cre-specific recombination events, respectively. (B) Southern blot analysis of four independently targeted ES clones before (wild-type/flox) and after (wild-type/null) Cre-mediated recombination. ES cell DNA was digested with EcoRV, subjected to agarose gel electrophoresis, and transferred to nitrocellulose. Fragments of 14.0 kb, 5.0 kb, and 3.2 kb corresponding to wild-type, floxed, and null alleles, respectively were detected by hybridization with a radiolabeled Cd151 genomic probe. (C) PCR analysis of genomic DNA from wild-type, heterozygous, and knockout mice using primers P1-P3. (D) Immunoblot analysis for the presence of Cd151 in whole cell lysates of MEFs isolated from wild-type and knockout mice.
Supplemental Figure 2 Normal skin morphology and inner ear function in Cd151<sup>−/−</sup> mice.

(A) No differences in skin organization between HE stained back skin samples of 8 month old wild-type and Cd151<sup>−/−</sup> mice. (B) Immunofluorescence analysis of ear and snout skin samples of Cd151<sup>+/+</sup> and Cd151<sup>−/−</sup> mice. The respective proteins are shown in green and red, yielding yellow upon colocalization. Nuclei are counterstained with TOPRO (blue). Both keratin 1 and 14 are normally localised in the suprabasal and basal epidermal layers. The epidermal-dermal border is not disrupted in Cd151<sup>−/−</sup> mice as basal keratinocytes nicely express b4 and are anchored to the basement membrane (laminin 10). (C) Mean absolute ABR thresholds and standard error (SEM) in dB sound pressure level for three 12 week old groups of mice (Cd151<sup>+/+</sup> , Cd151<sup>+/−</sup> , and Cd151<sup>−/−</sup>). Data show no statistically significant different hearing thresholds between the three groups (ANOVA, p > 0.05). E, epidermis; D, dermis; H, hair follicle; scale bars: 50 μm